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p63RhoGEF-mediated formation of a single polarized lamellipodium is required for chemotactic migration in breast carcinoma cells

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ABSTRACT

Short hairpin RNAs targeting 66 Rho-GEFs were screened for inhibition of chemotaxis. Six Rho-GEFs (p63RhoGEF, Trio, Duet, Net1, Frabin/Fgd4, and AAH33666) were found to be required for the serum-induced chemotactic migration of MDA-MB-231 human breast carcinoma cells. Knockdown of p63RhoGEF suppressed serum-induced RhoA activation and chemotaxis and caused the aberrant formation of multiple lamellipodial protrusions after serum stimulation while control cells formed a single polarized lamellipodium. These results indicate that p63RhoGEF plays a crucial role in serum-induced chemotaxis by limiting lamellipodial protrusion to one direction via RhoA activation.

1. Introduction

Chemotactic cell migration is essential for numerous physiological and pathological processes, including embryogenesis, organogenesis, immune responses, and tumor invasion and metastasis. The Rho family of small GTPases, including RhoA, Rac1, and Cdc42, are key regulators of actin cytoskeletal reorganization and play crucial roles in cell migration [1]. They are activated by Rho guanine nucleotide exchange factors (Rho-GEFs), which catalyze the conversion of Rho GTPases from the inactive GDP-bound form to the active GTP-bound form. In the human genome, there are approximately 70 Dbl-like Rho-GEF genes [2,3]. These Rho-GEFs contain a catalytic Dbl homology (DH) domain that is usually followed by a pleckstrin homology (PH) domain, but the regions outside the DH–PH domains are not conserved [2,3]. The large number of Rho-GEFs, compared with the small number (approximately 20) of Rho family GTPases, raises the possibility that each Rho-GEF selectively regulates specific functions of target Rho family GTPases. Thus, it is important to determine the functional roles and regulation mechanisms of each Rho-GEF in the context of various cellular processes.

p63RhoGEF was originally identified as a 63 kDa Rho-GEF that specifically activates RhoA [4]. An N-terminally truncated variant of p63RhoGEF, termed GEFT, was then identified and reported to activate Rac1 and Cdc42 [5], but later studies argued that GEFT also activates RhoA [6–9]. Several lines of evidence indicate that p63RhoGEF directly binds to the activated $G\alpha_{q/11}$, but not $G\alpha_{12/13}$, subunits of heterotrimeric G proteins and thereby functions downstream of $G\alpha_{q/11}$ to mediate the signaling pathways linking $G\alpha_{q/11}$-coupled receptors to RhoA activation [7–9]. Biochemical and crystallographic studies revealed that $G\alpha_q$ activates p63RhoGEF by relieving the autoinhibition of the catalytic DH domain by the PH domain [7–9]. Although the crucial role of p63RhoGEF in cellular responses induced by agonists of $G\alpha_{q/11}$-coupled receptors, such as angiotensin II- or endothelin-1-induced contraction of vascular smooth muscle cells, has become evident [10,11], little is known about its functional role in cell migration.

This study aimed to identify Rho-GEFs involved in serum-induced chemotactic migration in MDA-MB-231 human breast carcinoma cells. A screen of short hairpin RNAs (shRNAs) targeting 66 Rho-GEFs revealed that at least six Rho-GEFs (including p63RhoGEF) are required for the chemotactic response of MDA-MB-231 cells. The results also provide evidence that p63RhoGEF is involved in forming the single polarized lamellipodium in response to serum stimulation.

Abbreviations: DH, Dbl homology; FCS, fetal calf serum; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; PH, pleckstrin homology; RBD, Rho-binding domain; shRNA, short hairpin RNA

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2. Materials and methods

2.1. Cell culture and transfection

MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10% fetal calf serum (FCS). Cells were transfected with plasmids using Lipofectamine LTX (Invitrogen).

2.2. Plasmid construction

Expression plasmids encoding Rho(T19 N), Rac1(T17 N), and Cdc42(T17 N) were provided by K. Kaibuchi (Nagoya University). To construct the plasmid encoding YFP-p63RhoGEF, the cDNA for p63RhoGEF was amplified by PCR and inserted into the pEYFP-C1 vector (Clontech). The plasmid for GEF-inactive p63RhoGEF(L301E) [4] was constructed using a site-directed mutagenesis kit (Stratagene).

2.3. Construction of a Rho-GEF shRNA library

To construct a shRNA library targeting each of the human Rho-GEFs, candidate 19-nucleotide shRNA sequences were designed according to the reported method [12] and inserted into the pSUPER vector (Oligoengine). To assess knockdown, each shRNA plasmid was transfected into Jurkat cells together with the pNUL reporter plasmid, which encodes a chimeric cDNA composed of luciferase and the cDNA fragment (~400 base pairs) of the target gene, as reported [13]. If a shRNA construct effectively suppresses the expression of its target gene, luciferase expression will be reduced. Thus, the silencing effect of each shRNA was monitored by measuring luciferase activity. Using this reporter assay, one or two effective shRNA constructs targeting each of the 66 human Rho-GEFs were obtained (Supplementary Table S1). As a control shRNA, GL2 (5′-CCGTACGCGGAATACTTCGA-3′) or mutated SSH1 shRNA (5′-TCTTCCCCCAAGAAAGATA-3′) was used.

2.4. Cell migration assays

To screen Rho-GEF shRNAs for their effect on cell migration, MDA-MB-231 cells were cotransfected with a YFP plasmid and a Rho-GEF shRNA plasmid (with a molar ratio of 1:5) and cultured for 48 h. Cells were serum-starved for 3 h, suspended in serum-free medium, and then aliquots of 4×10^4 cells were loaded into the upper well of Transwell chambers (8 μm pore size, Corning). The lower wells were filled with DMEM/Ham's F12 medium, with or without 10% FCS. For chemokinetic migration assays, 10% FCS was added to both the upper and lower wells. After incubation for 4–8 h, cells were fixed with 4% formaldehyde and stained with 4′,6-diamidino-2-phenylindole. After the total number of cells was counted, the non-migrating cells on the top of the membrane were gently removed by wiping and rinsing, and the number of migrating cells on the lower face of the membrane was counted. The motility index was calculated as the percentage of migrating cells from the total number of cells.

2.5. Reverse transcription (RT)-PCR

To analyze the expression of Rho-GEF mRNAs, total RNA was isolated from MDA-MB-231 cells using the RNeasy Mini Kit (Qiagen) and reverse-transcribed to yield single-stranded cDNAs using the SuperScript Choice System (Invitrogen). The cDNAs were subjected to PCR amplification, as described [13]. The specific primers used for amplifying p63RhoGEF and GEFT cDNAs were 5′-GAATCCTATTCCATTGCGGG-3′ (p63RhoGEF-sense), 5′-GGGGTGCCCTGGTGAAAT-3′ (GEFT-sense), and 5′-TCTCCTCAGGCCACTGAC-3′ (p63RhoGEF/GEFT-antisense) [6]. The MegaMan Human Transcriptome Library (Agilent Technologies) was used as a control template to detect both transcripts.

2.6. Active RhoA and Rho-GEF pull-down assays

The active form of RhoA was analyzed by pull-down assays using the Rho-binding domain (RBD) of rhotekin fused to glutathione-S-transferase (GST), as described [14]. The active form of p63RhoGEF was analyzed by pull-down assays using RhoA(G17A), a guanine nucleotide-free form of RhoA, fused to GST, as described [15].

2.7. Immunoblotting

Cell lysates were subjected to immunoblotting as described previously [13]. Rabbit polyclonal antibodies against p63RhoGEF/GEFT (14839-1-AP; Proteintech) and GFP (A6455; Molecular Probes), and mouse monoclonal antibodies against RhoA (sc-418; Santa Cruz) and α-tubulin (B-5-1-2; Sigma–Aldrich) were purchased commercially.

2.8. Cell staining

Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min and permeabilized with PBS containing 0.1% Triton X-100. After washing with PBS, cells were stained with Alexa568-phalloidin (Molecular probes) to detect F-actin. Fluorescence images were obtained using a fluorescence microscope (DMIRBE, Leica).

3. Results

3.1. Identification of Rho-GEFs involved in the chemotactic migration of MDA-MB-231 cells

To identify Rho-GEFs involved in serum-induced tumor cell migration, Rho-GEF shRNAs were screened for suppression of serum-induced chemotactic migration in MDA-MB-231 breast carcinoma cells. Prior to the screening, the effect of expressing dominant-negative mutants of RhoA, Rac1, or Cdc42 on MDA-MB-231 cell chemotaxis was assessed. Chemotactic migration was analyzed using Transwell chambers where serum was added only to the lower chamber. Transfection of RhoA(T19N), Rac1(T17N), or Cdc42(T17N) significantly suppressed the serum-induced chemotactic migration of MDA-MB-231 cells (Fig. 1A), indicating that RhoA, Rac1 and Cdc42 are crucial for the serum-induced chemotactic response.

The effect of knocking down each Rho-GEF was then examined. Two independent shRNA plasmids targeting each of the 66 human Rho-GEFs were constructed, and silencing of target expression was assessed by luciferase reporter assays, as reported previously [13]. MDA-MB-231 cells were transfected with each Rho-GEF shRNA plasmid, cultured for 48 h, serum-starved for 3 h, and then subjected to chemotaxis assay. Of all the shRNA pairs targeting the 66 Rho-GEFs, six pairs, each targeting the Rho-GEF AAH33666, Duet, Frabin/FGD4, Net1, p63RhoGEF, or Trio, showed significant suppression of chemotaxis with each of the two shRNAs, while four pairs, targeting Rho-GEF FGD1, P-REX2, Tiam2, or α-Pix, showed significant suppression of chemotaxis with only one of the two shRNAs (Fig. 1B, Supplementary Table S2). RT-PCR analysis revealed that transcripts of the former six Rho-GEFs are expressed in MDA-MB-231 cells (Fig. 1C). These results suggest that at least these six Rho-GEFs (AAH33666, Duet, Frabin/FGD4, Net1, p63RhoGEF, and Trio) are involved in the serum-induced chemotactic migration of MDA-MB-231 cells. This study focused on the

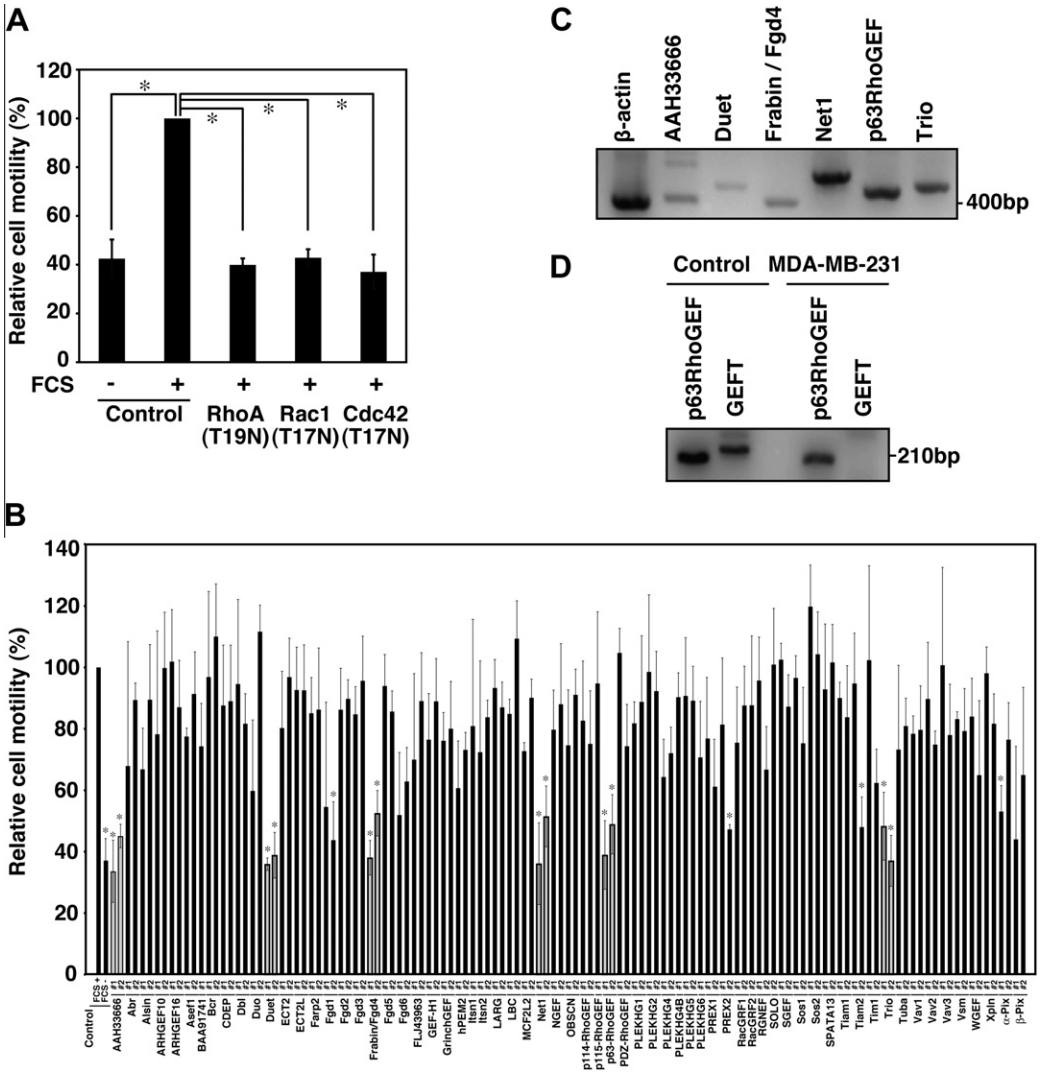

Fig. 1. Screening of Rho-GEFs involved in serum-induced chemotaxis. (A) Effect of RhoA(T19 N), Rac1(T17 N), and Cdc42(T17 N) on chemotaxis. MDA-MB-231 cells were transfected with plasmids, cultured for 24 h, serum-starved for 3 h, and subjected to chemotactic migration assays. (B) Effect of Rho-GEF shRNAs on chemotaxis. MDA-MB-231 cells were transfected with shRNA plasmids, cultured for 48 h, serum-starved for 3 h, and subjected to chemotaxis assays. In (A) and (B), data represent the means ± S.D. of three independent experiments, with the motility in serum-stimulated control cells set as 100%. *, $P < 0.01$. (C) Expression of Rho-GEF mRNAs in MDA-MB-231 cells. Total RNA from MDA-MB-231 cells was subjected to RT-PCR using specific primers designed to amplify PCR products of ~400 base pairs [13]. (D) Expression of p63RhoGEF and GEFT mRNA in MDA-MB-231 cells. RT-PCR analysis was performed as described previously [6]. A MegaMan Human Transcriptome Library was used as a control template.

role of p63RhoGEF, because of its prominent effect on cell polarity formation (see Fig. 5). p63RhoGEF has an N-terminally truncated isoform, termed GEFT [5,6]. RT-PCR analysis using specific primers for each isoform revealed that p63RhoGEF, but not GEFT, is expressed in MDA-MB-231 cells (Fig. 1D).

3.2. p63RhoGEF is involved in serum-induced RhoA activation

The role of p63RhoGEF in serum-induced RhoA activation was examined. Measurement of activated RhoA by GST-RBD pull-down assays revealed that serum stimulation induced RhoA activation and that ectopic expression of p63RhoGEF increased the levels of active RhoA in both serum-stimulated and unstimulated MDA-MB-231 cells (Fig. 2A). In contrast, expression of p63RhoGEF(L301E), a GEF-inactive mutant [4], suppressed serum-induced RhoA activation (Fig. 2A). These results indicate that p63RhoGEF catalyzes RhoA activation, as reported [4,6], and that p63RhoGEF(L301E) acts as a dominant-negative form. The effect of p63RhoGEF knockdown on serum-induced RhoA activation was then examined. Transfection of p63RhoGEF shRNA suppressed the expression of endogenous p63RhoGEF protein (Fig. 2B). GST–RBD pull-down assays revealed that knockdown of p63RhoGEF by shRNA significantly suppressed serum-induced RhoA activation in MDA-MB-231 cells (Fig. 2C). Similar results were obtained when we used another shRNA targeting p63RhoGEF (Supplementary Fig. S1A and B). These results indicate that p63RhoGEF is crucially involved in serum-induced RhoA activation. The serum-induced changes in the activity of endogenous p63RhoGEF was examined by pull-down assays using GST–RhoA(G17A), a guanine nucleotide-free form of RhoA known to bind specifically to the active form of Rho-GEFs [15]. The level of active p63RhoGEF increased 10 to 60 min after serum stimulation (Fig. 2D). These results indicate that p63RhoGEF is activated by serum and is involved in serum-induced RhoA activation in MDA-MB-231 cells.

3.3. Effect of p63RhoGEF overexpression on actin organization and cell morphology

The effect of p63RhoGEF overexpression on actin organization and cell morphology was then examined. MDA-MB-231 cells were transfected with YFP or YFP-p63RhoGEF, and stained with Alexa568-phalloidin to detect F-actin. Control YFP-expressing cells

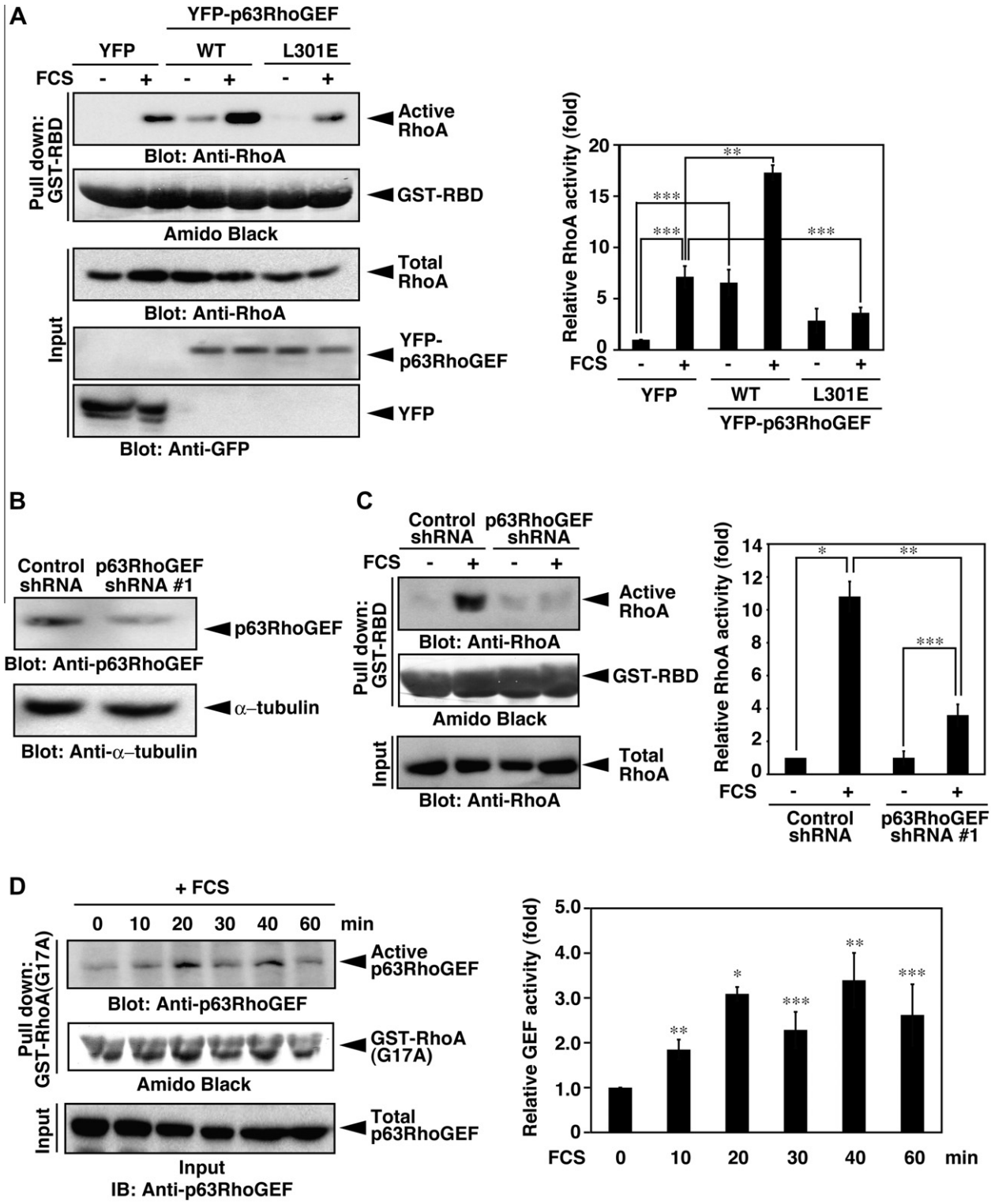

Fig. 2. p63RhoGEF is involved in serum-induced RhoA activation. (A) Effects of expression of p63RhoGEF (WT or L301E) on RhoA activity. MDA-MB-231 cells were transfected with YFP or YFP-p63RhoGEF (WT or L301E), cultured for 24 h, serum-starved for 3 h, and exposed to 10% FCS for 20 min. RhoA activity was measured by GST-RBD pull-down assays. Relative RhoA activity is shown as the mean ± S.D. of three independent experiments, with the activity in control cells in the absence of FCS set as 1.0. (B) Effect of p63RhoGEF shRNA on p63RhoGEF expression. MDA-MB-231 cells were transfected with control or p63RhoGEF shRNA #1, and cultured for 48 h. Cell lysates were immunoblotted with anti-p63RhoGEF antibody. (C) p63RhoGEF knockdown suppresses serum-induced RhoA activation. MDA-MB-231 cells were transfected with shRNA, cultured for 48 h, and analyzed as in (A). (D) Time course of p63RhoGEF activity. MDA-MB-231 cells were cultured for 24 h, serum-starved for 3 h and exposed to 10% FCS for the times indicated. The Rho-GEF activity of endogenous p63RhoGEF was measured by pull-down assays using GST-RhoA(G17A). Relative Rho-GEF activity is shown as the mean ± S.D. of three independent experiments, with the activity before FCS exposure set as 1.0. *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$.

mostly exhibited a polygonal morphology before serum stimulation but displayed a polarized cell morphology with a single F-actin-rich lamellipodial protrusion on one side of the cell after serum stimulation (Fig. 3A). Compared with control YFP, expression of YFP-p63RhoGEF often induced cell rounding and the formation of actin stress fibers both before and after serum stimulation (Fig. 3B). These

results further implicate p63RhoGEF in RhoA activation. Compared with the diffuse distribution of control YFP, YFP-p63RhoGEF was localized on the plasma membrane, as reported [16] (Fig. 3A and B).

3.4. Effect of p63RhoGEF knockdown on serum-induced chemotaxis and chemokinesis

To examine the role of p63RhoGEF in serum-induced cell migration further, the effect of p63RhoGEF knockdown on directional (chemotactic) and random (chemokinetic) migration was analyzed in MDA-MB-231 cells in response to serum stimulation. Serum was added only to the lower chamber for chemotaxis assays, whereas it was added to both the lower and upper chambers for chemokinesis assays. Knockdown of p63RhoGEF significantly suppressed the chemotactic migration of MDA-MB-231 cells toward serum; by contrast, knockdown of p63RhoGEF had no significant effect on the chemokinetic response of cells (Fig. 4; Supplementary Fig. S1C). These results suggest that p63RhoGEF plays a more specific role in chemotactic migration than chemokinetic migration.

3.5. p63RhoGEF is involved in the formation of the single polarized lamellipodium

To elucidate the mechanism by which p63RhoGEF knockdown impairs serum-induced chemotactic migration, the effect of p63RhoGEF knockdown on serum-induced changes in cell

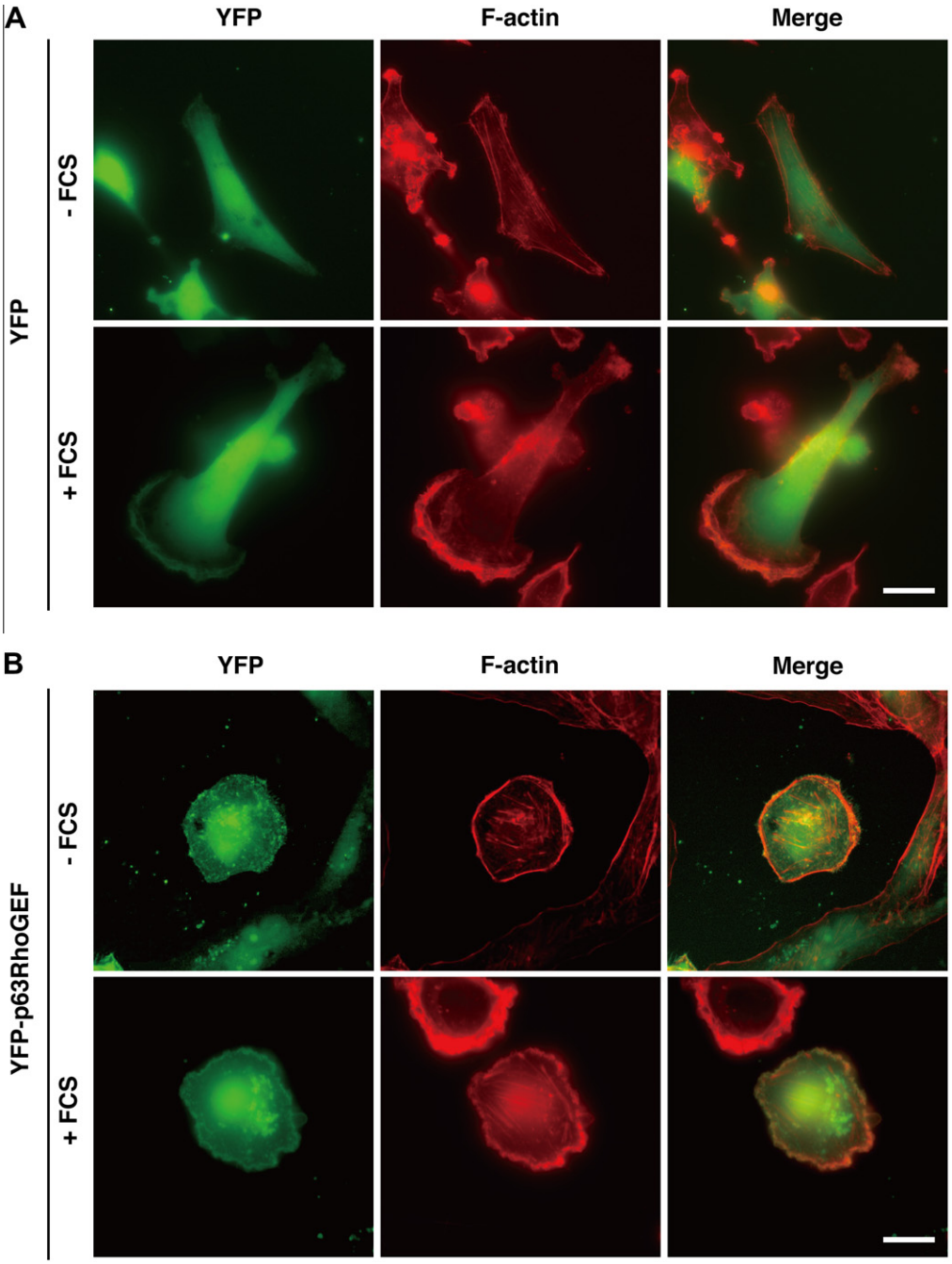

Fig. 3. Effect of p63RhoGEF expression on cell morphology and F-actin organization. MDA-MB-231 cells were transfected with control YFP (A) or YFP-p63RhoGEF (B). Cells were cultured for 24 h, serum-starved for 3 h, and stimulated with 10% FCS for 20 min (+ FCS) or left unstimulated (− FCS). Cells were fixed and stained with Alexa568-phalloidin to detect F-actin. Scale bar, 20 μm.

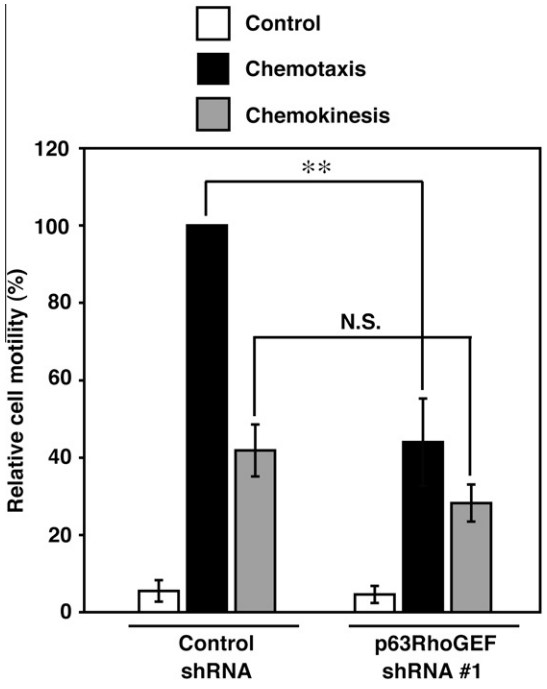

Fig. 4. Effect of p63RhoGEF knockdown on chemotaxis and chemokinesis. MDA-MB-231 cells were transfected with shRNAs, cultured for 48 h, serum-starved for 3 h, and subjected to chemotactic and chemokinetic migration assays. Data represent the means ± S.D. of three independent experiments, with the chemotactic migration of control cells set as 100%. **, $P < 0.01$; N.S., not significant.

morphology and actin cytoskeleton was analyzed. MDA-MB-231 cells were cotransfected with YFP and control shRNA or p63RhoGEF shRNA, and cell morphology and F-actin organization were analyzed by YFP fluorescence and Alexa568-phalloidin staining. As described above, control cells displayed a polarized cell morphology with a single lamellipodium after serum stimulation (Fig. 5A). This polarized cell morphology and single lamellipodium formation appear to support directional cell migration. Knockdown of p63RhoGEF had no apparent effect on the overall cell shape and F-actin organization before serum stimulation, but caused the production of multiple lamellipodial protrusions around the cell periphery after serum stimulation (Fig. 5A). Quantitative analysis showed that 36% and 11% of control shRNA cells displayed single and multiple lamellipodial protrusion(s), respectively, after serum stimulation. By contrast, 17% and 32% of p63RhoGEF shRNA cells displayed single and multiple lamellipodial protrusion(s), respectively, after serum stimulation (Fig. 5A). Similar results were obtained when we used another shRNA targeting p63RhoGEF (Supplementary Fig. S1D).

To examine the role of p63RhoGEF in polarized lamellipodium formation further, the effect of p63RhoGEF(L301E), a GEF-inactive p63RhoGEF mutant [4], was analyzed. Similar to the phenotype of p63RhoGEF knockdown, overexpression of p63RhoGEF(L301E) decreased the number of cells with single lamellipodium and increased the number of cells with multiple lamellipodia after serum stimulation (Fig. 5B and C). Taken together, these results suggest that p63RhoGEF plays a crucial role in serum-induced chemotactic migration by facilitating the formation of the single polarized lamellipodial protrusion in response to serum stimulation.

We also analyzed the effects of knockdown of the other five Rho-GEFs, identified in the screen, on serum-induced polarized lamellipodium formation. Duet and Trio shRNAs significantly decreased the number of cells with single lamellipodium and Duet shRNA increased the number of cells with multiple lamellipodia, compared with control shRNA, although their effects were weaker than those of p63RhoGEF shRNA (Fig. 5D). In contrast, knockdown of AAH33666, Frabin/Fgd4 or Net1 had no apparent effect on polarized lamellipodium formation. These results indicate that p63RhoGEF, Duet and Trio play a specific role in regulating serum-induced polarized lamellipodium formation.

4. Discussion

Using a shRNA library targeting 66 Rho-GEFs, six Rho-GEFs were found to be involved in serum-induced chemotaxis in MDA-MB-231 cells. Reflecting the essential role of RhoA, Rac1, and Cdc42 in chemotactic migration, the six Rho-GEFs identified in this study have distinct target specificity: p63RhoGEF, Net1, Duet (containing the C-terminal DH–PH domain of Kalirin), and the C-terminal DH–PH domain of Trio (Trio-C) activate RhoA, Frabin/FGD4 activates Cdc42, and the N-terminal DH–PH domain of Trio (Trio-N) activates Rac1 and RhoG [2,3,9,17,18]. The target of AAH33666 remains unknown. Trio and Net1 were reported to be involved in cell migration [17,19], but the roles of other Rho-GEFs in cell migration have yet to be elucidated. These six Rho-GEFs appear to play crucial roles in the chemotactic response to serum by coordinately regulating the activity of their respective target Rho family GTPases. Further studies on the signaling pathways and spatiotemporal regulation of each Rho-GEF activity will clarify the functional roles of individual Rho-GEFs and the mechanism of their coordination in chemotactic cell migration.

Of about 70 Rho-GEFs, the DH–PH domain of p63RhoGEF is most closely related to those of Trio-C and Duet [2,3]. All of these Rho-GEFs are activated by $G\alpha_q$ binding to the conserved C-terminal extensions of their PH domains [8,9]. Thus, they constitute a subfamily of Rho-GEFs that function as the $G\alpha_{q/11}$ effectors, which mediate RhoA activation downstream of $G\alpha_{q/11}$-coupled receptors. Intriguingly, all three Rho-GEFs were identified in our screen and knockdown of these Rho-GEFs affected polarized lamellipodium formation, suggesting that the signaling pathway of $G\alpha_{q/11}$-coupled receptor-mediated RhoA activation plays a pivotal role in the serum-induced chemotaxis and polarized lamellipodium formation of MDA-MB-231 cells. This hypothesis will be validated after identification of the agonist(s) in serum that induces the chemotactic response, and of their receptors.

Knockdown of p63RhoGEF suppressed cell migration, indicating that p63RhoGEF promotes cell migration. By contrast, prevention of $G\alpha_q$-mediated p63RhoGEF activation by MLK3 was reported to be required for cell migration [20]. These apparently contradictory results suggest that p63RhoGEF is indispensable for cell migration but its hyperactivation also impedes cell migration. Indeed, overexpression of p63RhoGEF or active RhoA(G14V) suppressed serum-induced chemotaxis (data not shown). Thus, it is likely that proper control of the levels of p63RhoGEF and RhoA activation is necessary for chemotactic migration.

RhoA is required for cell migration by generating contractile forces through ROCK-mediated myosin light chain activation [1]. In addition, previous studies showed that inhibition of RhoA signaling produces multiple competing lamellipodial protrusions that disrupt productive migration, indicating that RhoA signaling contributes to cell migration by suppressing inappropriate lamellipodial protrusions away from the leading edge [21,22]. Whereas Rac signaling promotes lamellipodium formation, RhoA signaling negatively regulates Rac activity [23]. Thus, inhibition of RhoA signaling may produce multiple lamellipodia through upregulation of Rac signaling. Our results showed that knockdown of p63RhoGEF or overexpression of GEF-inactive p63RhoGEF(L301E) induces multiple lamellipodia around the cell periphery after serum stimulation, suggesting that serum-induced p63RhoGEF activation plays

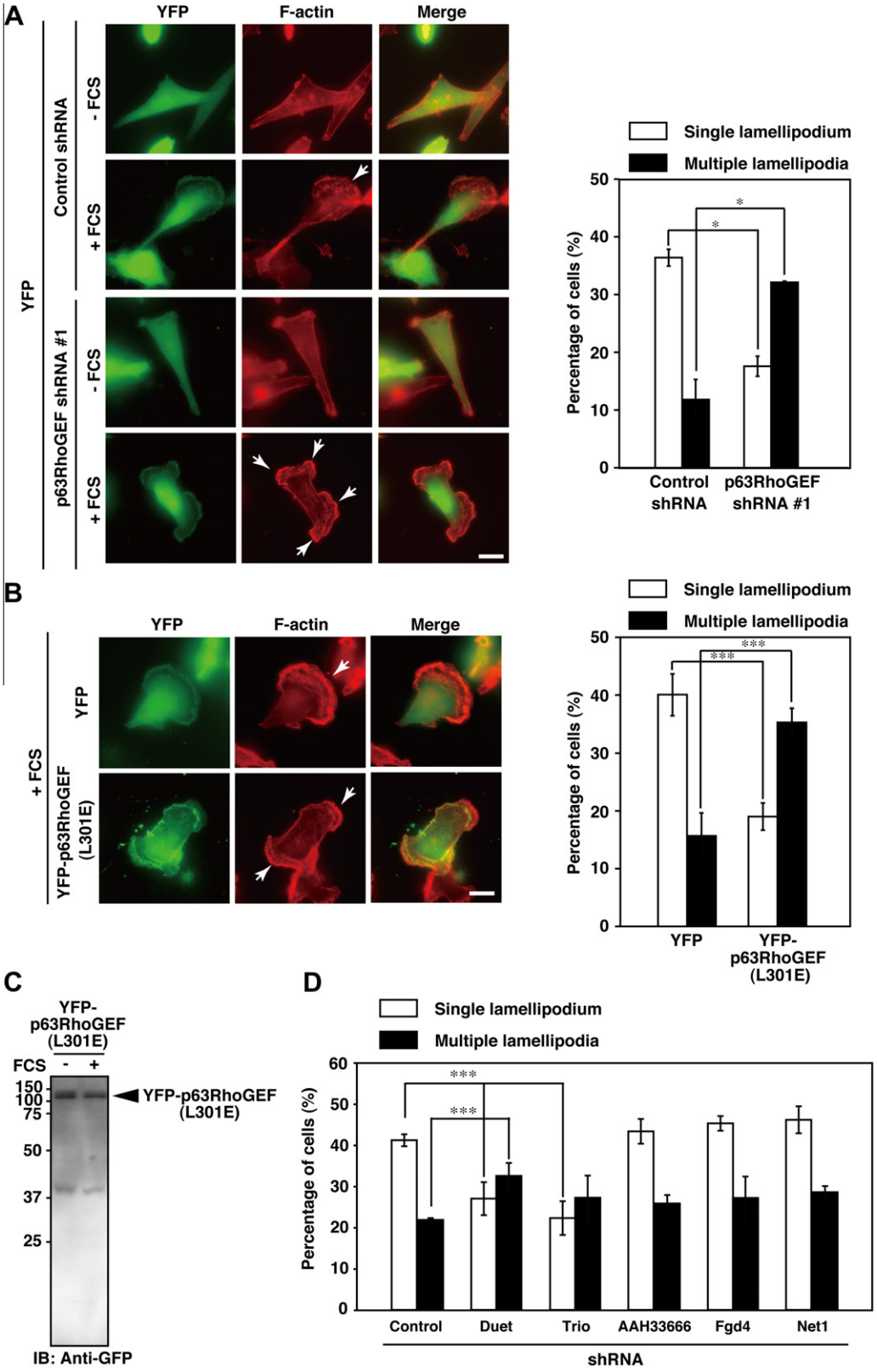

Fig. 5. p63RhoGEF is required for the formation of a single polarized lamellipodium. (A) Effect of p63RhoGEF knockdown on polarized lamellipodium formation. MDA-MB-231 cells were cotransfected with YFP and control or p63RhoGEF shRNA. Cells were cultured for 48 h, serum-starved for 3 h, and stimulated with 10% FCS for 20 min (+FCS) or left unstimulated (−FCS). Cells were fixed and stained with Alexa568-phalloidin. Right panel shows quantitative data. Cells treated with FCS were classified into three categories; cells with single lamellipodium (white bar), multiple lamellipodia (black bar), and no lamellipodium (not shown). (B) Effect of expression of p63RhoGEF(L301E) on polarized lamellipodium formation. Cells were transfected with YFP or YFP-p63RhoGEF(L301E) and analyzed as in (A). In (A) and (B), arrows indicate the positions of lamellipodial protrusions. Scale bar, 20 μm. (C) Immunoblot analysis of expression of YFP-p63RhoGEF(L301E). (D) Effects of knockdown of the other five Rho-GEFs on polarized lamellipodium formation. MDA-MB-231 cells were transfected with the indicated Rho-GEF shRNA and analyzed after serum stimulation, as in (A). Data represent the means ± S.D. of three independent experiments. *, $P < 0.001$; ***, $P < 0.05$.

a critical role in chemotactic migration by limiting the formation of the lamellipodium to the direction of cell movement and maintaining it at the leading edge.

The crucial role of RhoA in tumor cell migration and invasion has been well established [24,25], but it is difficult to target that specific function of RhoA. Identification of p63RhoGEF and related $G\alpha_{q/11}$-coupled Rho-GEFs as the essential RhoA activators for tumor cell migration provides potential therapeutic targets for suppressing RhoA-mediated tumor cell invasion and metastasis.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.01.043.

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